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Chromatin assembly controls replication fork stability

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During DNA replication, the advance of replication forks is tightly connected with chromatin assembly, a process that can be impaired by the partial depletion of histone H4 leading to recombinogenic DNA damage. Here, we show that the partial depletion of H4 is rapidly followed by the collapse of unperturbed and stalled replication forks, even though the S-phase checkpoints remain functional. This collapse is characterized by a reduction in the amount of replication intermediates, but an increase in single Ys relative to bubbles, defects in the integrity of the replisome and an accumulation of DNA double-strand breaks. This collapse is also associated with an accumulation of Rad52-dependent X-shaped molecules. Consistently, a Rad52-dependent—although Rad51-independent—mechanism is able to rescue these broken replication forks. Our findings reveal that correct nucleosome deposition is required for replication fork stability, and provide molecular evidence for homologous recombination as an efficient mechanism of replication fork restart.

Keywords: chromatin assembly; recombination; replication
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INTRODUCTION

A tight control of the DNA replication process is necessary to maintain the integrity of the genome, as impaired replication fork advance is associated with DNA damage and genetic instability. In eukaryotic cells, replicative stress induces a complex DNA damage response in which the S-phase checkpoints aim to maintain the stability of stalled replication forks (Lopes *et al*, 2001; Tercero & Diffley, 2001) and provide the time required for either to repair or tolerate the damage. In some cases, however, replication forks collapse, leading to double-strand breaks (DSBs) that are repaired by homologous recombination (HR).

Advance of the replication fork requires disruption of the chromatin fibre in front of the fork and assembly of the nascent DNA strands onto nucleosomes. DNA and histone synthesis are highly coordinated to ensure the exact supply of histones at the fork. Nucleosome assembly occurs rapidly after the passage of the replication fork and involves the initial deposition of the histone H3–H4 tetramer followed by the binding of two histone H2A–H2B dimers (Polo & Almouzni, 2006). DNA replication and chromatin assembly are tightly coupled by genetic and physical interactions between the DNA polymerase processivity factor proliferating-cell nuclear antigen (PCNA) and the PCNA-loader replication factor C (RFC) with chromatin assembly factor 1 (CAF1) and antisilencing factor 1 (Asf1; Shibahara & Stillman, 1999; Franco *et al*, 2005), two evolutionarily conserved histone chaperones that form part of the replication-dependent H3–H4 deposition complex (Tyler *et al*, 1999; Tagami *et al*, 2004).

The tight connection between chromatin assembly and DNA replication has led to the analysis of the consequences of mutations in genes encoding chromatin assembly factors on genetic instability. These studies have shown that defective histone H3–H4 deposition causes increased sensitivity to genotoxic agents, accumulation of DNA damage, activation of the S-phase checkpoints, and high rates of HR and chromosomal rearrangements (Tyler *et al*, 1999; Myung *et al*, 2003; Ye *et al*, 2003; Prado *et al*, 2004; Driscoll *et al*, 2007; Li *et al*, 2008). Another approach is the analysis of a yeast strain in which histone H4 is expressed from a doxycycline-inducible *tet* promoter (*t*::*HHF2* cells). Partial depletion of H4 in *t*::*HHF2* cells affects chromatin assembly and leads to an accumulation of recombinogenic DNA damage (Prado & Aguilera, 2005). Taken together, these results support the idea that impaired chromatin assembly affects genome integrity; however the mechanisms by which this genetic instability arises remain unclear.

RESULTS

Replication forks collapse in cells partly depleted of H4

To determine whether the accumulation of recombinogenic DNA damage in *t*::*HHF2* cells is a consequence of defective DNA replication, we investigated the fate of replication forks by using two-dimensional-gel electrophoresis. Cells were synchronized in G1 with α-factor and released into S-phase, and DNA samples were analysed at various times to follow the progression of replication forks from the early replication origin *ARS305* (Fig 1A). As shown in Fig 1B, firing and early elongation from *ARS305* can be followed by the formation of a bubble arc; replication fork progression along adjacent restriction fragments by the accumulation of a complete arc of single Y-shaped molecules; and converging forks and Holliday junction (HJ)-like structures by the accumulation of double Y- and X-shaped molecules. The total number of replication intermediates (RIs) at the origin was reduced in *t*::*HHF2* cells compared with the wild type (46 ± 10%; Fig 1C; data not shown) over time, although the proportion of bubbles, single Ys and Xs was not affected (Fig 1D). Similar results were

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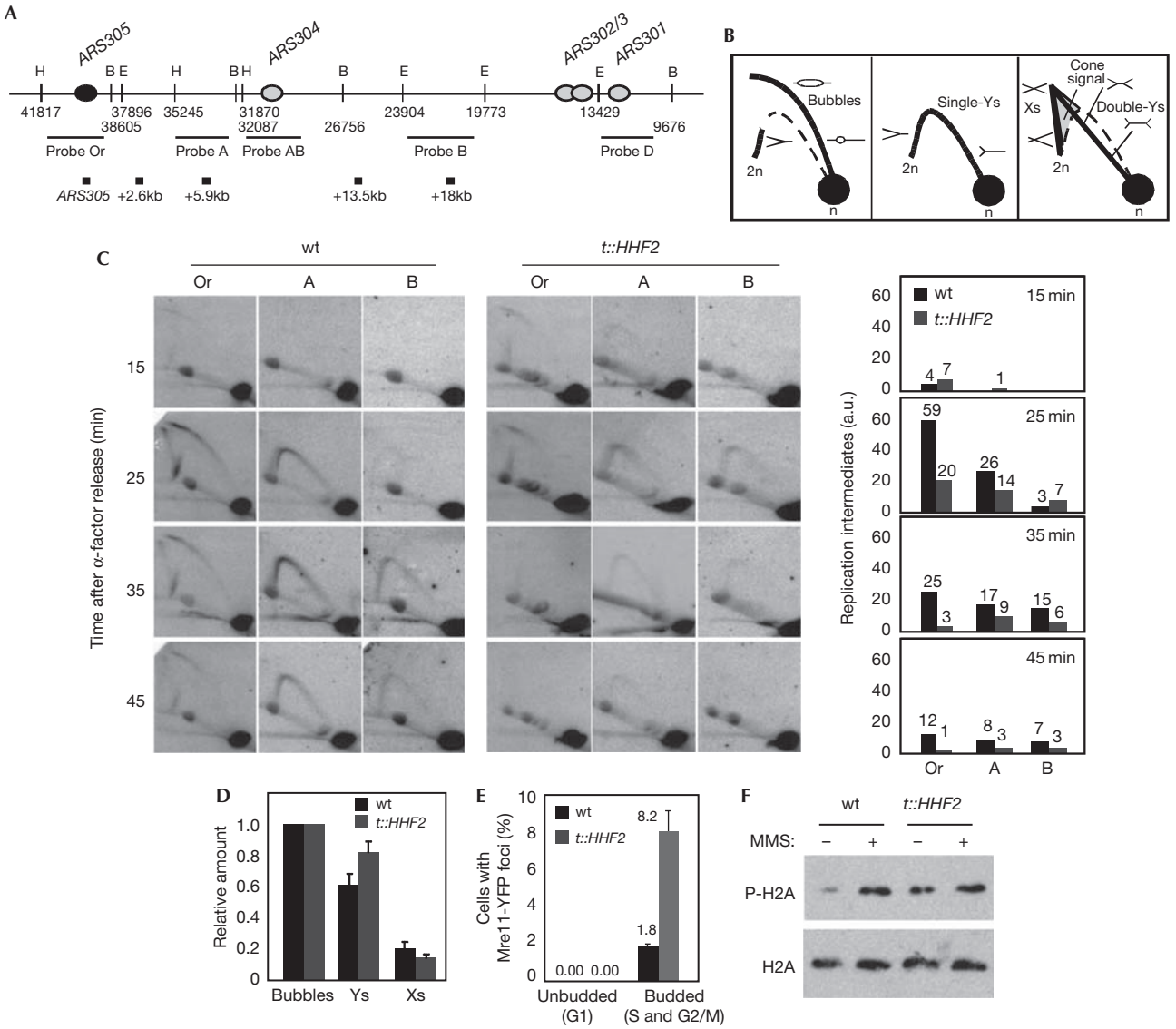

Fig 1 | Replication intermediates collapse in cells partly depleted of histone H4. (**A**) Schematic representation of the telomere-proximal region replicated from the early origin *ARS305* (black oval). The position of dormant origins (grey ovals), restriction fragments analysed by two-dimensional-gel electrophoresis and DNA fragments amplified by qPCR for ChIP analysis are shown. (**B**) Schematic representation of the migration pattern of the bubble-, single Y-, double Y- and X-shaped RIs by two-dimensional-gel electrophoresis. (**C**) Analysis of RIs at the *ARS305* (Or) and two adjacent regions (A and B) from DNA samples digested with *Eco*RV and *Hin*dIII. Quantification of the RIs, taking the total amount of wild-type RIs in the *ARS305* fragment at 1 h as 100 is shown. (**D**) Relative amount of bubbles, single-Y and X-shaped molecules at the *Eco*RV-*Hin*dIII *ARS305* fragment. (**E**) Percentage of cells with Mre11-YFP foci. (**F**) Determination by Western blot of the amount of total and phosphorylated histone H2A (P-H2A) in cells synchronized in G1 and released into S-phase for 1 h with 0.05% (+) or without (−) MMS. B, *Bam*H1; ChIP, chromatin immunoprecipitation; E, *Eco*RV; H, *Hin*dIII; MMS, methyl-methane sulphonate; qPCR, quantitative PCR; Or, origin; RI, replication intermediate; wt, wild type; YFP, yellow fluorescent protein.

obtained by the analysis of RIs from cells collected every 150 s upon G1 release and pooled into four samples to prevent the loss of RIs by asynchronic release from the α-factor arrest (data not shown). As most G1-synchronized *t::HHF2* cells replicated their DNA (see below), this decrease cannot be due to reduced replication origin firing, as, in this case, a complete single Y-arc, indicative of passive replication of the *ARS305* fragment by forks coming from a neighbouring origin in cells in which *ARS305* was not fired, should be detected. We have also eliminated the possibility that RIs break during DNA extraction by collecting and digesting the DNA in agarose plugs to preserve its integrity (supplementary Fig S1 online). Interestingly, RIs in the wild type peaked at 25 min in the origin and at 35 min in fragment B, whereas the peak of RIs in *t::HHF2* cells was at 25 min both in the origin and in fragments A and B, suggesting that the forks move faster in the mutant cells (Fig 1C). Consistent with an

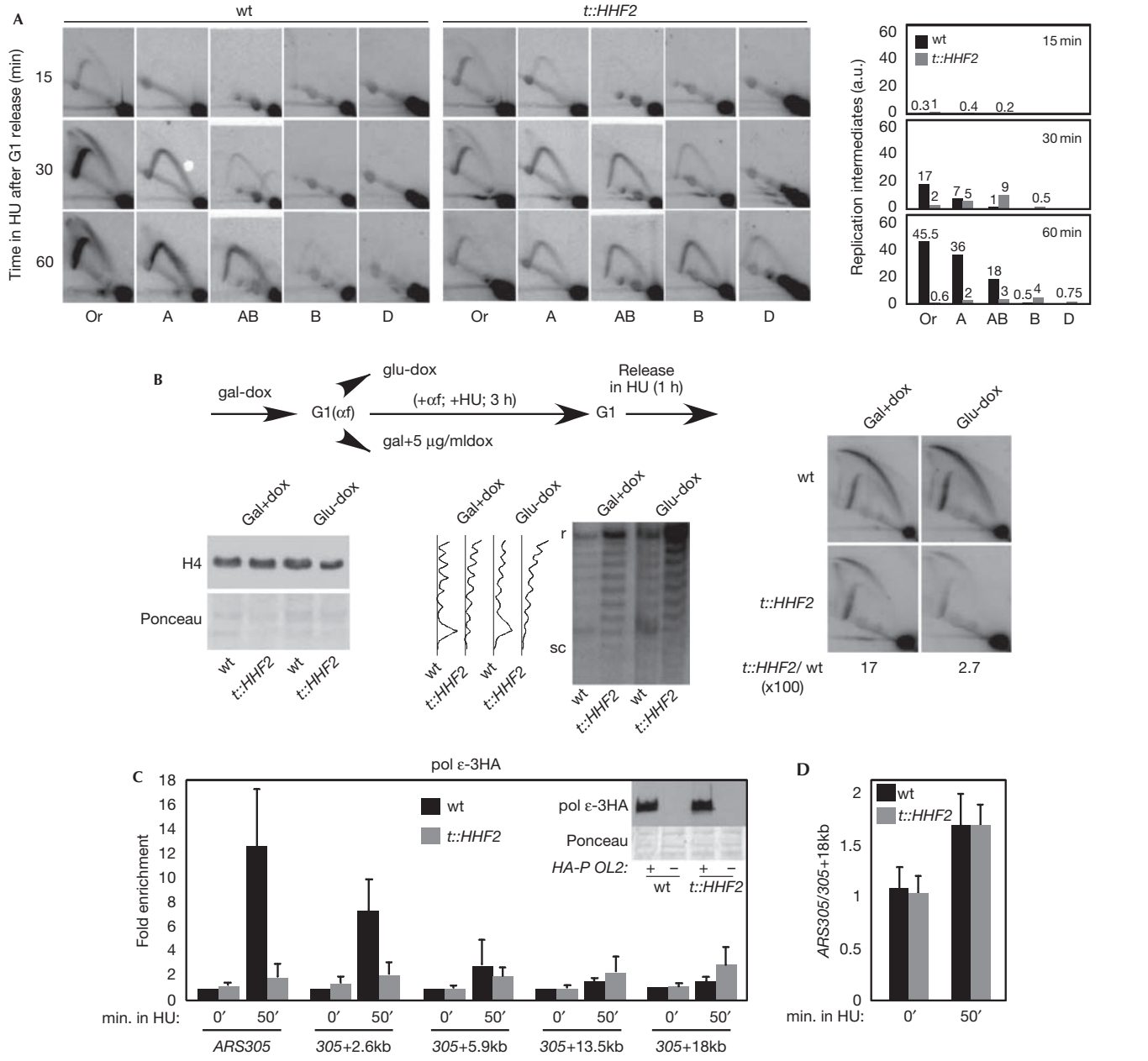

Fig 2 | Partial depletion of H4 causes the collapse of stalled replication forks. (**A**) Analysis of stalled RIs at the *ARS305* (Or) and four adjacent regions from DNA samples digested with *Eco*RV, *Hin*dIII and *Bam*HI. Quantification of the RIs, taking the total amount of wild-type RIs over the whole region at 1 h as 100, is shown. Similar results were obtained by synchronization with *cdc15-2* in telophase (supplementary Fig S2B online). (**B**) Analysis of the amount of H4, plasmid supercoiling and stalled RIs at the *Eco*RV-*Hin*dIII *ARS305* following depletion of H4. Ponceau staining is shown as a loading control; r and sc indicate relaxed and supercoiled plasmids, respectively. The percentage of RIs in *t:HHF2* cells relative to the wild type grown under the same conditions is shown. (**C**) Association of Pol ε-3HA to the replication fork in cells synchronized in G1 and released into S-phase in the presence of 0.2 M HU by ChIP analysis. Both input and immunoprecipitated DNA from G1 and HU-treated cells were amplified by real-time PCR with amplicons situated along the region (see Fig 1A). The enrichment in Pol ε-3HA at each zone is graphed relative to the enrichment in the wild type in G1, taken as 1. The inset shows the amount of Pol ε-3HA in wild-type and *t::HHF2* cells by Western blot. (**D**) Replication initiation from *ARS305* in HU-treated cells. The amount of DNA at *ARS305* relative to a fragment located at 18 kb from the origin, determined with the values obtained in (**D**) by amplification of the input with amplicons *ARS305* and *305* + 18 kb, is represented. ChIP, chromatin immunoprecipitation; dox, doxycycline; gal, galactose; glu, glucose; HA, haemagglutinin tag; HU, hydroxyurea; Or, origin; RI, replication intermediate; wt, wild type.

accumulation of broken replication forks in *t*::*HHF2* cells, we observed an increase in the amount of both Mre11-yellow fluorescent protein (YFP) foci and phosphorylation of histone H2A (P-H2A; Fig 1E,F, respectively), two of the earliest events in the DSB repair response.

Fork collapse is a direct effect of HY depletion

To determine the effect of partial depletion of H4 on the stability of stalled replication forks, G1 synchronized cells were released into S-phase in the presence of hydroxyurea (HU), a drug that reduces the pool of dNTPs and causes stalling of the replication forks. As shown by fluorescence activated cell sorter (FACS) analysis, wild-type and *t*::*HHF2* cells arrested at early S-phase to further slow progress as they were maintained in HU (see later). At the molecular level, wild-type replication forks initially stalled and accumulated in the proximity of the *ARS305* with a peak at 60 min after G1 release; then, as cells remained in the presence of HU, the signal in this region gradually disappeared and slowly increased in adjacent fragments (Fig 2A; supplementary Fig S2A online). By contrast, the forks appeared earlier and replicated more DNA in the mutant cells than in the wild type. Importantly, stalled RIs in the mutant cells did not accumulate and decreased progressively compared with the wild type (Fig 2A; supplementary Fig S2A online), and this reduction was associated with an increase in the amount of single Y- (twofold) and X-shaped (fivefold) molecules relative to bubbles (see later), further supporting that stalled replication forks collapse in *t*::*HHF2* cells. Consistent with this, the higher bubbles-dependent structural complexity of replicating relative to complete chromosomes was reduced in *t*::*HHF2* cells (supplementary Fig S3 online).

Next, we analysed the immediate effect of depleting H4 on replication fork stability (Fig 2B). For this, *t*::*HHF2* cells were transformed with plasmid pUK421 to increase the initial amount of H4 by expressing it from the inducible *tet* and *GAL1* promoters. Then, the cells were synchronized in G1 and split into two cultures—either expressing H4 or not—that were maintained in G1 with α-factor for 3 h and released into S-phase in the presence of HU for 1 h. At this point, *t*::*HHF2* cells in glucose showed a slight decrease in the total amount of H4, which, in turn, caused a rapid reduction in nucleosome density, as determined by a loss of plasmid negative supercoiling (Fig 2B; Kim *et al*, 1988). Importantly, this defect in nucleosome assembly was accompanied by a six fold reduction in the amount of stalled replication forks (from 17 to 2.7%; Fig 2B), indicating that fork collapse is a direct consequence of defective histone deposition.

Partial depletion of H4 causes a loss of replisome integrity

Next, using chromatin immunoprecipitation (ChIP) analysis we determined the association of the polymerase ε (Pol ε-3HA) with the fork in cells synchronized in G1 and released into S-phase in the presence of 0.2 M HU for 50 min (Fig 2C). As shown previously, in HU-treated wild-type cells, pol ε-3HA was enriched in the proximity of the *ARS305* (Fig 2C; Franco *et al*, 2005). By contrast, pol ε-3HA in *t*::*HHF2* cells was barely detected over the whole region, indicating that replication fork collapse is linked to defective replisome integrity. As shown for unperturbed replication forks, this reduction was not due to defective replication initiation, as determined by quantification of the total amount of DNA in the origin relative to an unreplicated fragment located at 18 kb from the *ARS305* both in G1 and S-phases with HU (Fig 2D).

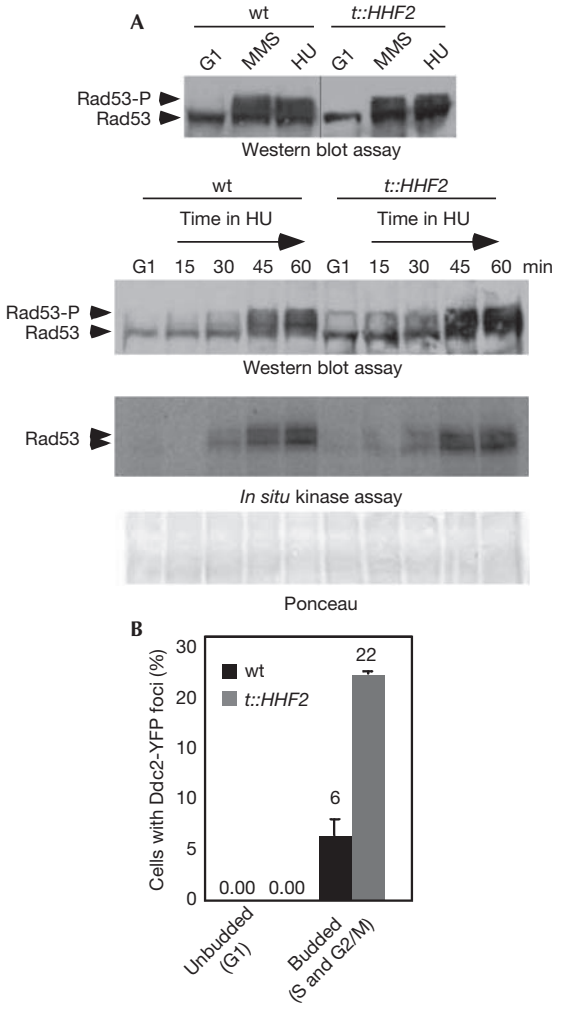

Fig 3 | The S-phase checkpoints are functional in *t*::*HHF2* cells. (**A**) Phosphorylation of Rad53 (Rad53-P) by Western blot and *in situ* kinase assay in cells synchronized in G1 and released into S-phase for various times in the presence of genotoxic agents (0.05% MMS or 0.2 M HU). (**B**) Percentage of cells with Ddc2-YFP foci. HU, hydroxyurea; MMS, methyl-methane sulphonate; wt, wild type; YFP, yellow fluorescent protein.

The S-phase checkpoints are functional in *t*::*HHF2* cells

The stabilization of stalled replication forks by S-phase checkpoints requires recruitment of the heterodimer Ddc2–Mec1 to damaged forks and the subsequent activation by phosphorylation of the Rad53 kinase (Lopes *et al*, 2001; Tercero & Diffley, 2001). As shown in Fig 3A, Rad53 was phosphorylated by the presence of replicative DNA damage to the same extent and with the same kinetics in *t*::*HHF2* and wild-type cells. In the absence of genotoxic agents, we observed a 3.7-fold increase in the frequency of budded *t*::*HHF2* cells with Ddc2-YFP foci compared with the wild type (Fig 3B). This result is consistent with the accumulation of P-H2A (Fig 1F), which is mediated predominantly

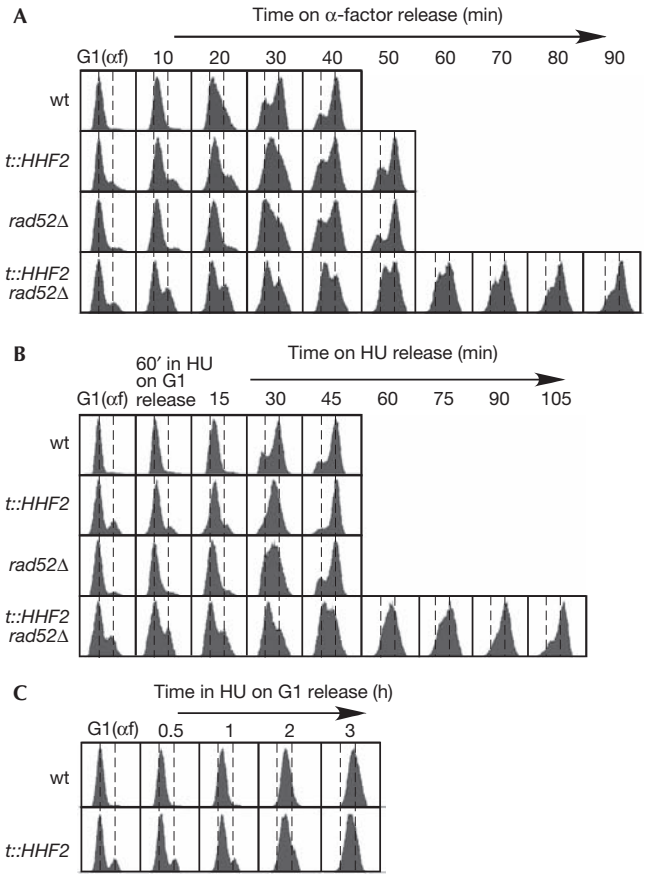

Fig 4 | Homologous recombination is required for DNA replication in *t*::*HHF2* cells. Cell-cycle progression by FACS analysis of cells synchronized in G1 and released into S-phase in (**A**) the absence of genotoxic agents, in (**B**) the presence of 0.2 M HU for 1 h and then released into fresh media, and in (**C**) the presence of 0.2 M HU. NCD was added upon the G1 (**A**) or the HU arrest (**B**) to prevent cells from re-entering a new cell cycle. FACS, fluorescence activated cell sorter; HU, hydroxyurea; NCD, nocodazole; wt, wild type.

by Mec1 (Downs *et al*, 2000). Therefore, replication fork collapse in *t*::*HHF2* cells is not due to defective S-phase checkpoints.

HR participates in the rescue of collapsed replication forks

It is noted that *t*::*HHF2* cells progressed through S-phase with only a slight delay compared with the wild type, as determined by DNA content analysis (Fig 4A; Prado & Aguilera, 2005). Furthermore, *t*::*HHF2* cells were able to resume DNA replication upon HU arrest (Fig 4B), and to adapt to a prolonged presence of HU (Fig 4C) without the loss of viability (supplementary Fig S4A online; Prado & Aguilera, 2005). Similar results were obtained with methyl-methane sulphonate (MMS; supplementary Fig S4B online). These data indicate that *t*::*HHF2* cells are endowed with efficient mechanisms to repair and restart collapsed forks.

The fact that *t*::*HHF2* cell viability is dependent on Rad52 (Prado & Aguilera, 2005) prompted us to evaluate the importance of HR in the rescue of broken forks. S-phase progression analysis using flow cytometry showed that the absence of Rad52 increased the time required for *t*::*HHF2* cells to complete DNA replication both under unperturbed conditions (Fig 4A) and on removal of the DNA damaging agent (Fig 4B; supplementary Fig S4B online). Interestingly, two-dimensional-gel analysis showed that the absence of Rad52 reduced the amount of RIs at the *ARS305* (47 ± 9%), whereas the double mutant *t*::*HHF2 rad52*Δ showed a synergistic decrease in the amount of RIs (8 ± 2%; Fig 5A; data not shown). As shown previously for *t*::*HHF2* cells, the shape of the Y-arc did not change in these mutants (Fig 5A), ruling out defects in replication origin firing. In addition, the absence of Rad52 had a minor effect on the stability of stalled replication forks both in wild-type and *t*::*HHF2* cells (Fig 5B; supplementary Fig S5A online), indicating that Rad52 did not participate in preventing replication fork collapse. Hence, these results suggest that HR is required for the rescue of collapsed forks. Consistent with this, the relative increase in X-shaped molecules was dependent on Rad52 (Fig 5B), suggesting that they represent HJ structures.

In conclusion, our results reveal the process of HR as an efficient mechanism for assisting collapsed replication forks. Interestingly, this mechanism does not require the DNA strand-exchange protein Rad51, as shown by the fact that *t*::*HHF2* viability was not affected by *rad51*Δ and the proportion of budded cells with Rad51-YFP foci was similar in *t*::*HHF2* and wild-type cells (Fig 5C).

DISCUSSION

The relevance of chromatin assembly in genetic instability has been evidenced from yeast to humans; however, the molecular mechanisms by which this instability takes place remains unknown. By using a yeast strain in which chromatin assembly can be impaired by the partial depletion of H4, we provide evidence that supports for the first time, to our knowledge, the idea that correct nucleosome assembly is required to maintain replication fork stability. Partial depletion of H4 causes a collapse of both unperturbed and stalled replication forks that is accompanied by a loss of integrity of the replisome, accumulation of DSBs and single Ys relative to bubbles, activation of the S-phase checkpoints and accumulation of Rad52-dependent X molecules. In addition, replication fork instability is increased in the absence of HR, further supporting the accumulation of broken replication forks in *t*::*HHF2* cells. This loss of fork stability is not due to defective S-phase checkpoints in *t*::*HHF2* cells. An alternative possibility is that defective histone deposition has a direct effect on the stability of the replication fork, as suggested by the fact that H4 depletion is rapidly followed by defects in nucleosome assembly and replication fork collapse. Consistent with this view, the binding of RFC, PCNA and polymerase ε to stalled replication forks (Franco *et al*, 2005; Han *et al*, 2007), and the stability of unperturbed and stalled RIs (M.C-R, and F.P., unpublished data) are affected in mutants defective in H3K56 acetylation—*asf1*Δ, *rtt109*Δ and *H3K56R*—a modification required for histone H3–H4 deposition during replication-dependent nucleosome assembly (Li *et al*, 2008).

As DNA synthesis and histone deposition are physically and genetically connected, it is possible that a reduction in the amount of available H4 uncouples both processes, exposing the forks to DNA breaks (Fig 5D). This possibility might explain the apparently faster advance of the forks in the mutant cells, which, in turn, could facilitate their collapse. Replication fork breakage would not

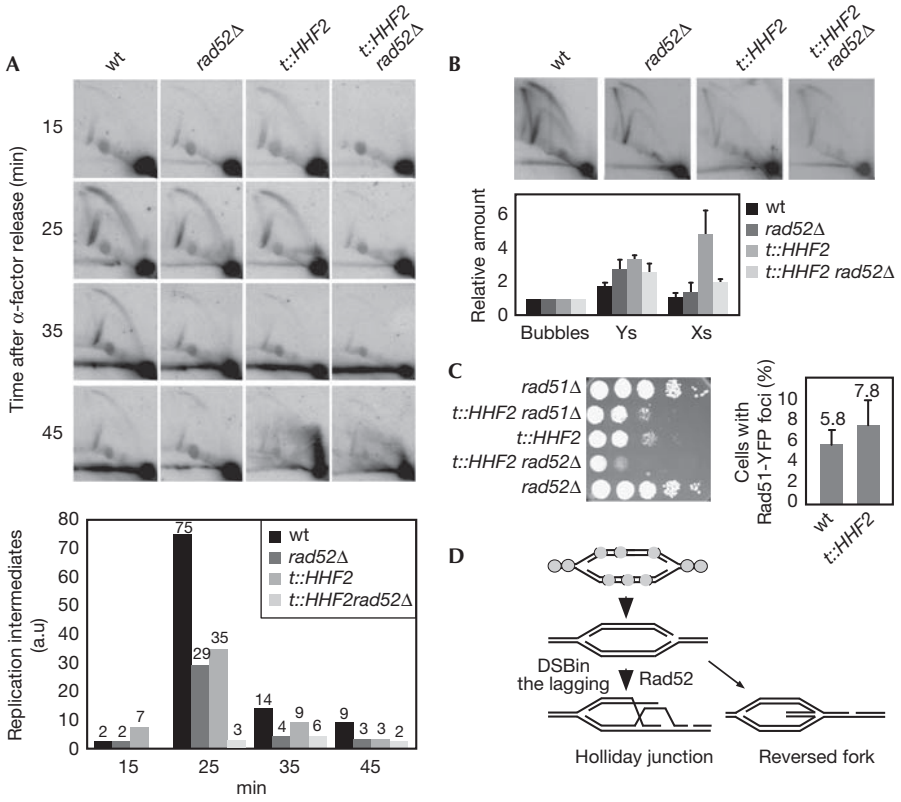

Fig 5 | Homologous recombination is required for replication fork progression in *t::HHF2* cells. (**A**) Analysis of RIs at the *Eco*RV-*Hin*dIII *ARS305* fragment of cells synchronized in G1 and released into S-phase at various times. Quantification as detailed in Fig 1C. (**B**) Relative amount of bubbles, single-Y and X-shaped molecules at the *Eco*RV-*Hin*dIII *ARS305* fragment from cells synchronized in G1 and released into S-phase in the presence of 0.2 M HU. To obtain a more reliable quantification, the DNA was extracted with a modified version of the CTAB protocol that increases the stability of the X-shaped molecules (R.E. Wellinger, personal communication), even though similar results were obtained with the CTAB protocol (supplementary Fig S5B online). (**C**) Effect of *rad51*Δ on *t::HHF2* viability, and the percentage of wild-type and *t::HHF2* cells with Rad51-YFP foci. (**D**) Model for the collapse and rescue of replication forks in *t::HHF2* cells. DSB, double-strand breaks; HU, hydroxyurea; RI, replication intermediate; wt, wild type; YFP, yellow fluorescent protein.

only convert bubbles into single Ys, but also explain the accumulation of DSBs and the activation of the S-phase checkpoints.

We have shown that HR is required for the repair of broken forks in *t::HHF2* cells. In principle, the substrate for HR could be the one-ended DSB generated by collapse of the fork or, alternatively, the two-ended DSB generated on the arrival of the oncoming replication fork. The latter possibility seems to be unlikely because the progressive appearance of RIs in our kinetics indicates that replication forks progressed only from the *ARS305* to the telomere. Our results are more consistent with the accumulation of one-ended DSBs that are repaired by a recombination mechanism in which the broken end invades the sister chromatid (Fig 5D). This could occur either by annealing of the parental or the nascent strands. We favour the former possibility because it would lead to HJs, which are dependent on Rad52 (Prado *et al*, 2003) and migrate in the 2n spike, whereas the latter would lead to reversed forks that are dependent on the helicase Rad5 (Blatyak *et al*, 2007)—which is not required for *t::HHF2* viability (data not shown)—and migrate in the cone of two-dimensional gels (Fig 1B). The annealing promoted by Rad52 would provide a 3′-end to prime new DNA synthesis and reinitiate replication, which could be associated or not with the assembly of a true replisome. Finally, the *t::HHF2 rad52*Δ mutant is sick but still viable and is not affected by the absence of the DNA annealing protein Rad59 (data not shown), indicating that other mechanisms are also able to deal with broken forks in the absence of HR.

An interesting implication of the proposed mechanism is that the break has to occur at the lagging chain of the fork to provide a parental 3′-end (Fig 5D), suggesting that this chain might be more sensitive to defects in histone deposition. Whether this is related to the fact that some inverted DNA repeats form hairpin structures at the lagging template, which cause checkpoint-blind fork stalling (Voineagu *et al*, 2009), is an interesting possibility that remains to be addressed. In any case, mutations that affect the lagging strand polymerase (Pol δ) but not the leading strand polymerase (Pol ε) result in an accumulation of Rad52-dependent, Rad51-independent HJs (Zou & Rothstein, 1997). This result also supports our observation that the rescue of broken forks by HR does not require Rad51. We hypothesize that the more open chromatin structure at the fork might bypass the requirement for strand exchange proteins, as supported by the fact that Rad51 is dispensable for HR when the chromatin of the donor is in an open conformation (Sugawara *et al*, 1995).

The fact that repression of histone genes causes DNA synthesis inhibition in human cells (Nelson *et al*, 2002) suggests that the effect of defective histone deposition on replication fork stability might be conserved, even though this inhibition is in contrast with the efficiency of *t*::*HHF2* cells to complete DNA replication. This could be related to a higher efficiency of HR in yeast, but might also reflect the more demanding structural and topological package of DNA into chromatin in mammalian cells.

METHODS

Yeast strains, growth conditions, plasmids, and standard methods for DNA extraction and detection, plasmid supercoiling, flow cytometry, detection of YFP constructs, Western blot and *in situ* kinase assay are included in the supplementary information online.

Analysis of RIs. Cell cultures were arrested with sodium azide (0.1% final concentration) and cooled down on ice. Total DNA was extracted using the CTAB protocol at the indicated times—except for Fig 5B and supplementary Fig S1 online, which were extracted using the G2/CTAB protocol and in agarose plugs, respectively—digested with restriction enzymes, resolved by neutral/neutral two-dimensional-gel electrophoresis as described previously (Brewer & Fangman, 1987) and analysed by sequential hybridization of the same membrane with various probes. Quantification of the RIs was normalized to the total amount of DNA, including linear monomers, to the size of the restriction fragment and to the percentage of cells synchronized either in G1 (α-factor) or in telophase (*cdc15-2*). Kinetics in Figs 1C, 2A and 5A were repeated 2–4 times with similar results. The average and standard deviation of 3–5 independent experiments are shown in Figs 1D, 5B and supplementary Fig S5B online.

Chromatin immunoprecipitation. ChIP assays were performed as described previously (Hecht & Grunstein, 1999) with the HA mouse monoclonal antibody 12CA5 (Roche; www.roche.com). Oligonucleotide sequences for the real-time PCR amplifications performed on purified DNA before (input) or after (immunoprecipitated) immunoprecipitation are shown in supplementary Table SII online. Pol ε-3HA enrichment at each specific region was calculated as the ratio between the immunoprecipitated and the input relative to the same ratio in the wild type arrested in G1, taken as 1. The average and standard deviation of three independent experiments are shown.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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